MTDH Promotes Cancer Stem Cell Phenotypes and Correlated with Immune Infiltration in Hepatocellular Carcinoma

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Research Article

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Abstract

Purpose
MTDH (Metadherin) has been suggested as one of the key oncogenes in most cancer types, including hepatocellular carcinoma. The aim of this study was to investigate the role of MTDH in hepatocellular carcinoma on stemness and immune infiltration.

Methods
MTDH expression in HCC tissues was detected using TCGA and GEO databases. Immunohistochemistry was used to analyze tissue samples. MTDH was stably knocked down or overexpressed by transfection with lentivirus in two HCC cell lines. Invasive and migratory abilities were evaluated using matrigel invasion and wound healing assays. Western blotting and qRT-PCR were used to determine gene expression. Flow cytometry, immunofluorescence, and tumor sphere formation assays were used to identify stem-like cell characterization. MTDH inhibition was evaluated in vivo for its effects on tumor growth. The correlation of MTDH with immune cells, immunomodulators, and chemokines was analyzed through the ssGSEA and TISIDB databases.

Results
The expression of MTDH is increased in hepatocellular carcinoma and leads to poor prognosis. HCC cells overexpressing MTDH invaded and migrated more, exhibited a stem cell-like phenotype, and formed spheres. MTDH inhibition attenuated these effects. In vivo, inhibition of MTDH suppressed HCC progression and expression of CD133. MTDH was positively correlated with immature dendritic cells, Th2 cells, central memory CD8 T cell, memory B cell, and CXCL2. MTDH was negatively associated with activated CD8 T cell, eosinophil, activated B cell, monocyte, CX3CL1, and CXCL12.

Conclusions
High levels of MTDH expression in HCC patients are associated with poor prognosis, promoting tumour stemness, immune infiltration and HCC progression.

Introduction
Primary liver cancer is one of the six most common cancers and the third leading cause of cancer death in the world. 75%-85% of primary liver cancer cases are caused by hepatocellular carcinoma (Sung, Ferlay et al. 2021). The complex pathogenesis of liver tumour, postoperative recurrence, and drug resistance, the therapeutic effect is still not ideal (Kim, Lee et al. 2018). Metastasis and high recurrence rate are important reasons for the poor prognosis of patients with this disease. So, it is crucial to understand the
relevant mechanisms involved in hepatocellular carcinoma and find new therapeutic approaches and targets.

According to many studies, a small proportion of tumor cells, including Hepatocellular carcinoma (HCC) and colorectal cancer, are capable of self-renewing, proliferating, and differentiating, these cells are called cancer stem cells (CSCs) (Wang, Wu et al. 2018, Rhee, Chung et al. 2020). Liver CSCs are closely related to tumorigenesis, metastasis, cell proliferation, and postoperative recurrence. Surface markers of CSCs in HCC include CD133, CD90, and EpCAM. High expression levels of markers of CSCs presented increased stem cell characteristics and tumour sphere-forming capacity (Liu, Kong et al. 2015, Naijin Xu, Xiezhao Li et al. 2018, Cao, Zhao et al. 2019). There is an infiltration of multiple immune cells in hepatocellular carcinoma, including T lymphocytes (Barsch, Salie et al. 2022), B cells (Garnelo, Tan et al. 2017), dendritic cells (DCs) (Suthen, Lim et al. 2022), and natural killer (NK) cells (Ducimetiere, Lucchiari et al. 2021). The microenvironment of hepatocellular carcinoma is formed by these tumor-infiltrating immune cells. The type and level of immune cells not only have prognostic value but also influence the response to immunotherapy.

There is also MTDH known as AEG-1 (astrocyte elevated gene-1) or LYRIC (lysine-rich CEACAM1). MTDH has been suggested as one of the key oncogenic genes in most cancer types (Sarkar and Fisher 2013). In malignant tumors, MTDH promotes the proliferation capacity (Chen, Jia et al. 2018), migration (Zhu, Dai et al. 2011), cell survival, and angiogenesis (Luni Emdad, Seok-Geun Lee et al. 2009), as well as a poor prognosis, for instance, lung cancer, prostate cancer, and breast cancer. The importance of MTDH in HCC has been demonstrated in numerous studies (Zhang, Zhangyuan et al. 2021, Chen, Dong et al. 2022), whether the expression of MTDH can induce the stem cell characteristics of HCC and its effect on HCC immune cell infiltration remains unclear.

As compared to normal liver tissue, liver cancer showed high levels of MTDH expression. MTDH expression was associated with a poorer prognosis. Our research showed that MTDH was expressed at higher levels in tumor spheres than in adherent cells. MTDH expression in HCC cells correlates positively with CD133, Oct4 and Nanog expression levels, which are related to stem cells. Furthermore, inhibition of MTDH expression was able to inhibit tumor growth. Our study confirmed that MTDH is related to immune cell infiltration with the ssGSEA method and the TISIDB database.

**MATERIALS AND METHODS**

**HCC Samples**

All of the gene expression profile files as well as the clinical information were obtained from two public databases. The Cancer Genome Atlas database (TCGA, https://portal.gdc.cancer.gov/) contains the transcriptional gene expression in human cancer and healthy tissues. In this study, after removing the samples with incomplete clinical information, a total of 369 liver tumour tissues and 50 healthy liver tissues were used for analysis. In the Gene Expression Omnibus (GEO, www.ncbi.nlm.nih.gov/geo/),
GSE14520 (the sequencing platform is GPL3921) deleted incompletely informed samples, and 210 tumor samples and non-cancer pairs were used for analysis. All sample clinical information was presented in Table 1 for the datasets. And, the liver tumor tissue microarrays of 9 paired samples (HLivH030PG03) were purchased from SHANGHAI XINCHAO (Shanghai, China).

**Differential expression analysis and survival analyzed**

Data in TCGA converted to TPM values for subsequent analysis. For GSE14520, perform data normalization correction and log2 transformed. Convert probe IDs into gene symbols using the platform annotation file. Genes with multiple probes were expressed using their maximum values. After data preprocessing was performed, followed by differential expression analysis and statistics using R. A survival curve analysis was performed using the R packages "Survival" and "Survminer", with the best cut-off value for MTDH calculated for both datasets. R packages “ggstatsplot” and “corrplot” were used for data visualization.

**Cell**

We purchased Huh7 cells from the Chinese Academy of Sciences Cell Bank, as well as MHCC-97H cells from the Liver Cancer Institute at Fudan University's Zhongshan Hospital. In an incubator with 5% carbon dioxide, all cells were cultured in Dulbecco's modified Eagle medium (DMEM) (10% Foetal bovine serum (FBS)), 100 units/mL penicillin, and 100 units/mL streptomycin.

**Sphere formation assay**

Tumor spheres were cultured in SFM consisting of 20 ng/mL EGF (PeproTech), DMEM/F12 (HyClone), 20 ng/mL bFGF (PeproTech), and 20µL/mL B27 (Gibco). Single cells (1x10⁴) were planted in a 6-well ultra-low adsorption plate (Corning) containing 1.5mL of serum-free culture medium. All cells were incubated for 14 days in incubators with 5% CO2 at 37°C. Inverted microscopes were used to count tumor spheres larger than 50 mm.

**Extraction of proteins and western blotting assay**

To prepare the protein from cells, cells were lysed in RIPA solution (CW BIO) containing protease inhibitor cocktail solution. Determination of protein concentration in solution with BCA kit (Beyotime). Proteins were separated by SDS-PAGE (Beyotime Millipore) and transferred to PVDF membranes (Millipore). PVDF membranes were closed with 5% skim milk at room temperature (RT) for one hour, then bound overnight at 4°C to the primary antibody. Addition of 0.1% Tween20 to tris-buffered saline (TBST) containing 0.1% Tween-20 was used as a buffer for washing three times and for incubating the membranes with 1:5000 secondary antibodies (EarthOx) for one hour. Then, after washing three times with TBST, each time for five minutes, blots were visualized with chemiluminescence reagents (Beyotime). The antibodies were MTDH (CST, 14065T), CD133 (YT5192, ImmunoWay), Nanog (CST, 4903T), and GAPDH (YM3215, ImmunoWay).

**Total RNA extraction and qRT-PCR**
Trizol (Takara) was used to extract RNA from cells, and complementary DNA (cDNA) composition was performed utilizing the PrimeScript™ RT Reagent Kit (TaKaRa). QRT-PCR was performed using SYBR Premix ExTaq (TaKaRa). We used CFX96 Real-Time PCR Detection System (Bio-Rad) to perform QRT-PCR reactions. A PCR reaction was conducted using primers from Sangon Biotechnology (Shanghai, China). The 2-ΔΔCt method was used to calculate the data. Table 2 shows primer sequences.

Cell transfection

To obtain an MTDH stable overexpression and suppression cell line, cell lines were infected with lentivirus. The MTDH overexpression lentiviruses and MTDH-RNAi lentivirus vectors and NC lentivirus vectors were acquired from GeneChem (Shanghai, China). On the previous day, 3 × 10^4 cells were grown in each well of a six-well plate. When cells grown to 30% were transfected according to the manufacturer’s protocols with a multiplicity of infection (MOI) of 5. After 96 hours, fluorescence microscopy and WB were used to verify the transfection efficiency. Then, puromycin screening was applied for 2 weeks (GeneChem).

Immunofluorescence

Cells were planted in a 24-well plate. A 4% paraformaldehyde solution was used to fix the cells for 20 minutes after incubation for 24 hours. The cells were treated with 0.3% TritonX-100 (Sigma) for cell membrane permeabilization for fifteen minutes. Anti-Nanog antibody was incubated overnight with the cells at 4°C after 120 min incubation in 5% BSA in TBST. Next, the cells were washed 3 times with Phosphate-buffered saline (PBS) and incubated with DyLight 649 AffiniPure Goat Anti-Rat IgG (1:200, Abbkine) for 1 hour in a dark humidified box. The final counterstaining was done using DAPI (4',6-diamino-2-phenylindole) for 10 min. Photographed with a fluorescent microscope (Nikon).

A wound-healing test

We cultured MHCC-97H and Huh7 cells in six-well plates. When the cell density in the wells reached 90%, subsequent experiments were performed. After scratching the cells with a 200ml pipette, 2% FBS cell culture medium was used for continued culture. All cells were incubated with 5% carbon dioxide in an incubator at 37°C. A microscope was used to take photographs of the wounded areas.

Invasion and migration assay

The ability of cells to migrate or invade was assessed by 24-well transwell chambers covered with or without Matrigel (BD Biosciences). The supplements were kept in 5% carbon dioxide at 37°C for 12 hours. Then, 2x10^5 cells were resuspended in 200 µl of Serum-free cell cultures (SFM) and placed in transwell cups with an 8-micron pore membrane (BD). Simultaneously, cells culture medium containing 10% FBS was added to the lower layer for a total of 600 µl. The chambers were maintained in 5% carbon dioxide for 24 hours at 37°C. The cells that fail to pass through the pores and remain in the upper chamber were carefully wiped with a cotton swab. Afterward, migrating cells were fixed in 4% paraformaldehyde solution for 20 minutes. Then, the cells remaining in the chambers were stained in 200 µl 0.1% crystal violet for 30 min and followed by using a microscope to count in five random areas.
Flow cytometry

Cells were prepared and washed with PBS after trypsin digestion. Then, cells were suspended in 80 µL PBS and added with 2 µL PE-CD133 antibody (Miltenyi Biotec) and FcR Blocking Reagent 20 µL (Miltenyi Biotec). Under light-proof conditions, the mixture was incubated at 4°C for 10 minutes. Next, all cells were washed in PBS three times, followed by resuspension with 500 µl of PBS. The mixture was tested using a FACS Calibur Flow Cytometer (BD Biosciences).

Animal experiments

The experimental BALB/c-nu were obtained from the Animal Experiment Center of Chongqing Medical University. Experiments were conducted in the animal facilities of the Animal Experiment Center of Chongqing Medical University (12-hour light/dark cycle, 27 ± 2°C, 50 ± 10% humidity). The nude mouse were randomly divided into 2 groups of 3 mice each. MHCC-MTDH-LV cells (2×106) were resuspended in a complete culture medium. Then, 100µL of cell suspension were injected subcutaneously in nude mouse (female, 4–6 weeks old). The tumors were detached after 4 weeks. Next, the tumor volume calculation formula is length × width² / 2. And the tumor tissues were submitted to IHC analysis. The study was approved by the Ethics Committee of the Second Hospital of Chongqing Medical University.

Immunohistochemical staining

All slides were dewaxed and hydrated gradually. Antigen extraction with Citrate buffer under high pressure and high-temperature conditions. All slides were rinsed with PBS and then closed with goat serum for 10 min at RT. Then, the slides were exposed to anti-MTDH and anti-CD133 at 4°C overnight. Subsequently, HRP-labeled streptavidin was added, followed by the DAB reagent. tap water was dehydrated with gradient ethanol. The slides were washed with xylene 2 times and wrapped with neutral balsam. The protein expression level was assessed with ImageJ software. The average optical density was calculated by measuring the IOD (integrated optical density) and area of each image, which reflects the concentration per unit area of the target protein.

ssGSEA

The infiltration abundance of 28 immune cell species in TCGA samples was quantified using ssGSEA. Gene set data for immune cells were downloaded from the website (https://www.cell.com/cms/10.1016/j.celrep.2016.12.019/). To compare immune cell differences between groups, box plots were constructed. The R package used was “GSVA”(Hanzelmann, Castelo et al. 2013), “ggplot2”.

TISIDB Database

TISIDB is an online analysis site for the analysis of tumor-immune interactions(http://cis.hku.hk/TISIDB/) (Yu, Wang et al. 2012). To further investigate the immunological impact of MTDH in cancer, we have analyzed and evaluated the TISIDB database through the "Immunomodulators" and “chemokine modules".
Statistical analysis

All experiments were repeated at least 3 times. Data were analyzed using R 4.1.2 and GraphPad Prism. Student’s t-test was used to compare the two groups of continuous variables. The Kruskal-Wallis test and the Wilcox test were used for non-parametric tests. The criterion of statistically significant was $P < 0.05^*$, $P < 0.01^{**}$, $P < 0.001^{***}$, $P < 0.0001^{****}$.

RESULTS

MTDH is upregulated in HCC and positively related to poor prognosis.

To identify the aberrant expression of MTDH in HCC, we downloaded the microarray gene profiling data from GEO (GSE14520/GPL3921) and TCGA. In the 50 cases of normal liver tissues and 369 cases of liver cancer tissues from TCGA MTDH mRNA expression was demonstrated to be upregulated in liver tumour tissues. Similarly, in GSE14520, MTDH expression was also increased in the tumour in GSE14520 (n = 420) (Fig. 1A, B). Next, the expression of MTDH was examined in human HCC tissues (n = 9) and para-cancerous (n = 9) by IHC. According to the results, HCC tissues overexpressed MTDH protein compared with para-cancerous tissues (Fig. 1C). Furthermore, we constructed the Kaplan-Meier curve of HCC using "Survival" and "Survminer" R packages. Shorter survival times were associated with higher levels of MTDH (Fig. 1D, E). There is a higher expression of MTDH in liver cancer tissues than in normal liver tissue or in para-cancerous tissues, which affects the prognosis for patients with liver cancer.

MTDH promotes HCC cell migration and invasion.

We transfected two cell lines, Huh7 and MHCC-97H, with related lentiviruses, blank control (NC) vs. Overexpression (OE) and blank control (NC) vs. Knockdown (RNAi), to obtain stable overexpression and suppression of expression of the cells. Then, WB experiments were used to verify the MTDH protein expression after transfection (Fig. 2A). A migration and invasion assay showed that MTDH overexpression significantly accelerated MHCC-97H and Huh7 cell migration and invasion (Fig. 2B). Conversely, cells with MTDH knockdown exhibited less potential for migration and invasion (Fig. 2C). Accordingly, the wound healing assay confirmed that MTDH overexpression significantly promoted wound healing, whereas MTDH knockdown suppressed scratch wound healing in HCC cells (Fig. 2D). These experiments indicate that the high MTDH expression correlates positively with the ability of HCC cells to invade and migrate.

MTDH is associated with the CSC phenotypes in HCC

To test whether MTDH is related to the stem cell phenotype, our study examined MTDH and HCC stemness using the TCGA database. TCGA data show a significant positive correlation between HCC tissue MTDH expression and CD133, Nanog, and Oct4 expression (n = 369) (Fig. 3A). Our previous research confirmed that Liver cancer stem cells (LCSCs) could be enriched by using serum-free stem cell medium to promote tumour sphere formation. Next, we performed serum-free stem cell culture in MHC-
97H and Huh7 cells, followed by Western blot and quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays determine MTDH expression in stem cell spheres and attached cells. Compared to liver cancer adherent cells, LCSCs expressed more MTDH and stem cell markers CD133, Oct4 and Nanog than liver cancer adherent cells at the expression level of mRNA (Fig. 3B). Results showed that both MTDH, CD133 and Nanog were higher than liver cancer adherent cells at the protein expression level (Fig. 3C). These results showed that MTDH is highly expressed in LCSCs.

**Overexpression of MTDH enhances the liver CSC phenotypes.**

To confirm that MTDH maintains stem-like phenotypes within HCC cells, stem cell markers were detected in MHCC-97H and Huh7 cell lines overexpressing MTDH (CD133, Oct4, Nanog). Through PCR experiments, we demonstrated that the markers of CSCs expression were higher in MTDH-overexpressing cells at the mRNA compared to the NC groups (Fig. 4A). MTDH-overexpressing Huh7 and MHCC-97H cells also expressed more CD133 and Nanog protein (Fig. 4B). A sphere culture assay showed the formation of more spheres in MTDH-overexpressing Huh7 and MHCC-97H cells than in control cells (Fig. 4C). Next, flow cytometry showed that the overexpression of MTDH expression effectively increased the numbers of CD133 + HCC cells (Fig. 4D). In addition, MTDH overexpression effectively increased the CSC phenotypes in HCC cells.

**Knockdown of MTDH inhibits liver cancer stem cell phenotype.**

The PCR results demonstrated that the stem cell markers’ expression levels were lesser in MTDH-RNAi cells at the mRNA compared to the NC group (Fig. 5A). In contrast to the NC group, MTDH-RNAi showed lower levels of CD133 and Nanog expression (Fig. 5B). Downregulation of MTDH reduced the number of both MHCC-97H and Huh7 cell spheres compared to the control LV-NC group in a sphere culture assay (Fig. 5C). Nanog fluorescence intensity was greater in the NC group than in the MTDH-RNAi group in cell immunofluorescence experiments (Fig. 5D). Taken together, the above experimental results confirm that inhibition of MTDH expression attenuates the acquisition of hepatocellular carcinoma stem cell phenotype.

**MTDH knockdown reduces tumor growth and CD133 expression in vivo**

We injected male BALB/c nude mice with LV-NC, LV-MTDH-RNAi MHCC-97H cells to test for MTDH’s impact on tumour growth. We showed that the MTDH-RNAi group exhibited a smaller tumor volume than the 97H-NC group, confirming that MTDH promotes tumour growth (Fig. 6A, B). The typical HE-stained images of nude mouse tumor tissue are shown (Fig. 6C). To further clarify the role of MTDH in tumour stem cell phenotype, CD133, and MTDH proteins were detected by IHC in tumor tissues of nude mice. We found that CD133 was decreased with MTDH low expression (Fig. 6D). Thus, these results further provide that MTDH overexpression promotes liver tumor growth and CSC phenotype.
MTDH Expression and Immune cell infiltration

A significant association was found between immune cell infiltration and survival in HCC. Using the ssGSEA method, we quantified immune cell infiltration scores in HCC samples in TCGA to understand the relationship between MTDH and infiltration. Firstly, the results demonstrated a significantly higher infiltration of activated B cell, immature B cell, memory B cell, activated CD8 T cell, gamma delta T cell, effector memory CD8 T cell, central memory CD8 T cell, effector memory CD4 T cell, eosinophil, immature dendritic cell, MDSC, monocytes, macrophage, mast cell, monocyte, neutrophil, natural killer (NK) cells, natural killer T cell, and T helper cells (Th) in healthy tissue than in HCC (Fig. 7A), suggesting that immune cells are essential in the progression of HCC. Based on the median MTDH expression, we divided the liver cancer samples in TCGA into MTDH high expression and MTDH low expression groups and then assessed the immune cell infiltration in both groups. Notably, the levels of memory B cell (p < 0.0001), immature dendritic cell (p < 0.001), Th2 cell (p < 0.001), central memory CD4 T cell (p < 0.01) infiltration were higher in the MTDH high expression group. Meanwhile, in the MTDH low expression group, activated CD8 T cell (p < 0.0001), macrophage (p < 0.01), activated B cell (p < 0.01), effector memory CD8 T cell (p < 0.01), mast cell (p < 0.01), eosinophils (p < 0.05), monocyte (p < 0.05), Th1 cell (p < 0.05) levels were significantly increased (Fig. 7B).

We then examined the relationship between immune cell expression levels and MTDH expression levels (Fig. 8A). The results showed that immature dendritic cell (r = 0.28, p < 0.0001) (Fig. 8B), Th2 cell (r = 0.26, p < 0.0001) (Fig. 8C), memory B cell (r = 0.25, p < 0.0001) (Fig. 8D), central memory CD4 T cell (r = 0.22, p < 0.0001) (Fig. 8E), central memory CD8 T cell, natural killer T cell, activated dendritic cell, activated CD4 T cell, and natural killer cell have also shown a positive relationship with MTDH. Activated CD8 T cell (r = -0.23, p < 0.0001) (Fig. 8F), eosinophils (r = -0.13, p < 0.05) (Fig. 8G), activated B cell (r = -0.12, p < 0.05) (Fig. 8H), monocyte (r = -0.12, p < 0.05) (Fig. 8I), macrophage, mast cell have been shown to be negatively correlated with MTDH.

Correlation of MTDH expression with immunomodulators and chemokines

Immune checkpoint inhibitors (ICIs) are gaining increasing attention as a tumour immunotherapy strategy in different types of cancer, helping some patients to improve their prognosis. MTDH and the various immunosuppressive agents in the TISIDB database did not show a significant correlation in our online analysis (Fig. 9A). However, in the analysis of the correlation with immunostimulants (Fig. 9B), a positive correlation was found between MTDH and MICB (r = 0.207, p = 5.76e-05) (Fig. 9C), NT5E (r = 0.201, p = 9.8e-05) (Fig. 9D), and TNFSF14 (r = 0.143, p = 0.00576) (Fig. 9E). Based on the results above, MTDH may be involved in the regulation of tumour immunity.

Cell migration is induced by chemokines and their receptors. There is a correlation between the expression of MTDH and the expression of chemokines and receptors in immune cells based on data from the TISIDB database (Fig. 10A, B). In hepatocellular carcinoma, MTDH expression levels were
positively correlated with CXCL2 ($r = 0.224, p = 1.34 \times 10^{-5}$) (Fig. 10C) and negatively correlated with CX3CL1 ($r = 0.245, p = 1.73 \times 10^{-6}$) (Fig. 10D) and CXCL12 ($r = 0.208, p = 5.4 \times 10^{-5}$) (Fig. 10E). However, for chemokine receptors, MTDH did not correlate significantly with them.

**DISCUSSION**

This study demonstrated that MTDH expression was higher in HCC tissues than in the normal liver, and was associated with shorter survival time, stronger migration, and invasive ability. Next, we focused on the effects of MTDH on stemness acquisition and immune infiltration of hepatocellular carcinoma cells. MTDH could promote stemness enhancement in HCC cells, and high expression of MTDH may resist cancer immunotherapy. This bioinformatics analysis and basic findings will provide additional information for further understanding of the prognosis and treatment of HCC patients.

Differential expression analysis, immunohistochemistry, and survival analysis confirmed that MTDH expression was upregulated in hepatocellular carcinoma. A high level of MTDH expression was associated with a shorter survival time in patients with hepatocellular carcinoma. Meanwhile, after correcting for TNM staging of LIHC data samples in the TCGA database, multifactorial Cox proportional risk analysis suggests that low MTDH expression reduced the risk of death. (Zhang, Huang et al. 2021) These results disclosed that MTDH can be used as a valid poor prognostic biomarker to identify HCC with poor clinical prognosis. Additional studies revealed that MTDH also participates in proliferation, tumour progression, invasiveness (Tokunaga, Nakashima et al. 2014), and metastasis (Chen, Jia et al. 2018). We performed invasion, migration, and scratch assays on hepatocellular carcinoma cell lines overexpressing and knockdown MTDH. The results obtained were consistent with those of Yoo BK et al (Yoo, Emdad et al. 2009). The above results suggest that MTDH plays a role in the progression of HCC.

CSCs are capable of promoting metastasis, and enhancing resistance to tumor therapy, including liver cancer (Chen, Hsu et al. 2021). CD133, Nanog, Oct4, and many other molecular markers related to the maintenance of stemness in human CSCs have been reported (Liu, Kong et al. 2015, Vlashi and Pajonk 2015). CD133 has been as a surface marker for LCSCs. Co-expression of Nanog and OCT4 is related to aggressive behavior of tumor and worse clinical outcome in HCC cells. A study by Bin Hu et al. found strong correlations between MTDH and CD133 and SOX2 in gliomas (Hu, Emdad et al. 2017). The contribution of MTDH to tumor stem cell phenotype in HCC is unclear. We explored the correlation between MTDH and stem cell markers of HCC by correlation analysis and experimental studies. All three stemness markers in the TCGA LIHC dataset were positively correlated with MTDH. Meanwhile, MTDH expression was experimentally shown to be increased in hepatocellular carcinoma spheres. Overexpression of the MTDH gene in HCC cells enhanced self-renewal abilities increased the proportion of CD133 + cells, and promoted the expression of tumor stemness markers. After knocking down MTDH, however, stem cell markers were expressed less and self-renewal was suppressed in cells. We performed tumorigenic experiments in nude mice. The primary tumor size was reduced in MTDH inhibited group with decreased CD133 protein expression. The results confirmed that MTDH affects the stem of
hepatocellular carcinoma. Thus, in our studies, we demonstrated that MTDH promotes an increase in tumor stem cells in hepatocellular carcinoma cells, which may lead to a worse prognosis.

It has been shown that MTDH can influence the Wnt/β-catenin, Ha-ras, and PI3K/Akt pathways. The Wnt/β-catenin pathway maintains the CSC phenotype. (Mao, Fan et al. 2014) In gastric cancer, MTDH forms a complex with β-Catenin and LEF1 to promote β-catenin protein translocation occurrence and activate genes downstream of Wnt signaling (Pandit, Li et al. 2018). CD133 glioma cells overexpressing MTDH maintain stemness, and drug resistance through Wnt/β-catenin protein signaling (Hu, Emdad et al. 2017). The precise mechanism of MTDH regulation of CSC in HCC will be further explained by experiments and bioinformatics analysis in the future.

Tumor progression and treatment depend on immune cell infiltration. Mature DCs can induce specific immune responses in the body, acting as anti-infective and anti-tumor agents. Conversely, immature DCs can inhibit the function of antigen-specific effector T cells in the body to further induce immune tolerance. Different subpopulations of CD4+ T helper cells (Th) exist, include Th1, Th2, and Th17 cells. It has been shown that Th2 cytokines (IL-4 and IL-10) promote tumor growth and metastasis, while Th1 cytokines (IL-2 and TNF-α) are associated with a good prognosis in HCC. (Mantovani, Allavena et al. 2008, Zhou, Chong et al. 2009) We found that A high level of MTDH expression was positively correlated with an increase in Th2 cells and immature DCs. We speculate that MTDH may increase immune tolerance and metastasis of tumor cells by regulating the infiltration level of immature DC cells or Th2 cells, which in turn leads to poor prognosis in patients. In contrast, it negatively correlated with the infiltration levels of activated CD8 T cell cells, eosinophils, activated B cells, and monocyte. As a result of these findings, MTDH plays a critical role in regulating the tumor immune infiltration in HCC.

MTDH was significantly correlated with immunostimulants (MICB, NT5E, and TNFSF14). Chemokines initiate lymphocyte infiltration early in the development of malignancies in order to enhance the activity of antitumor agents. Gradually, chemokines have been found to reduce apoptosis, promote proliferation, enrich CSCs in tumors, and increase the resistance of tumor cells to therapy. (Morein, Erlichman et al. 2020) Analyzing the TISIB database revealed a positive correlation between MTDH expression levels and CXCL2 expression levels, whereas a negative correlation was found between MTDH expression levels and CXCL12 and CX3CL1 expression levels. CXCL2 has been shown to promote the invasion and migration of hepatocellular carcinoma cells. (Lu, Li et al. 2016) The recurrence rate of intrahepatic or extrahepatic metastases is lower in HCC patients that express high levels of CX3CL1 and its receptor CX3CR1. (Matsubara, Ono et al. 2007) Human lung microvascular endothelial cells (HPMEC) are attracted to suspension cultured cells by MTDH via the CXCR4/CXCL12 axis, suggesting that MTDH may promote hepatocellular carcinoma cell metastasis via CXCR4/CXCL12. (Zhou, Deng et al. 2014) Based on our analysis, MTDH expression was negatively correlated with CCXCL12, but not significantly with CXCR4. This contradicts our results, and exploring the interactions and mechanisms between CCXCL12 and MTDH will help to further explain the role of MTDH in HCC.
There are still some limitations to this study. The exact mechanism by which MTDH affects hepatocellular carcinoma stem cells needs to be studied in vivo and in vitro. In addition, we found that MTDH expression is closely related to immune infiltration and prognosis. The exact mechanisms of immune infiltration of MTDH need to be explored further.

**Conclusions**

In summary, high expression of MTDH is associated with poor prognosis in HCC. HCC progression may be influenced by MTDH by regulating tumor stemness and immune infiltration, which provides additional evidence for the possible function of MTDH as a potential molecular marker for HCC.

**Abbreviations**

HCC  Hepatocellular carcinoma;
CSCs  Cancer stem cells;
DMEM  Dulbecco's modified Eagle medium;
FBS  Foetal bovine serum;
LCSCs  Liver cancer stem cells;
MOI  multiplicity of infection;
SFM  Serum-free cell cultures;
TBST  Addition of 0.1% Tween20 to tris-buffered saline;
PBS  Phosphate-buffered saline;
QRT-PCR: quantitative reverse transcription polymerase chain reaction

**Declarations**

**Author contributions:** Yiying Wang and Meimei Shen completed the experiments and wrote the manuscript. Jian Gao helped to revise the manuscript. All authors agreed on the final manuscript.

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Conflict of Interest

No conflict of interest has been declared by any of the authors.

Institutional animal care and use committee statement: All animal experiments comply with internationally recognised principles for the care and use of laboratory animals (2023 (24), The Ethics Committee of the Second Hospital of Chongqing Medical University).

Data availability

This study uses publicly available data sets for analysis and links to them are included in the article.

References


liver tumorigenesis by restricting hepatocyte-driven macrophage activation and inflammation.


Figures
Figure 1

MTDH overexpression has been linked to a worse prognosis in HCC. MTDH mRNA expression in normal liver tissues (normal) compared with liver cancer tissues (cancer) (A, B). Images of normal and cancer tissues stained with MTDH by IHC (scale bar=200 μm). MTDH protein levels in human liver cancer tissues (n = 9) were remarkably higher than those in para-cancerous (n = 9) by IHC analysis, **P < 0.01 (C). Patient's overall survival (OS) curves according to MTDH expression (D, E). *P < 0.05, **P < 0.01.
Figure 2

The MTDH promotes proliferation, migration, and invasion of HCC cells. In Huh7 and MHCC-97H cells, Western blotting revealed the efficacy of MTDH overexpression and knockdown (A). Characteristic images of transwell invasion assays 24h after planting (B, C). Typical pictures of scratch width at 0 hours, and 48 hours after scratch in cells photographed under inverted microscopy (D). *P < 0.05, **P < 0.01 and ***P < 0.001.
MTDH correlates with the stemness properties in HCC. Correlation between MTDH and CD133, Nanog, Oct4 in TCGA(A). Through qRT-PCR, expression levels of MTDH, CD133, Nanog, Oct4 were measured in attached cells and tumour spheres from 97H and Huh7 cell lines. In tumor spheres, all four genes were expressed at increased levels(B). MTDH, CD133, and Nanog were higher in attached cells than in tumour spheres (C). Gene GAPDH serves as the internal reference. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
Figure 4

MTDH overexpression promoted stem cell phenotypes and self-renewal in HCC cell lines. By PCR (A) and Western blot (B), we determined MTDH expression and stemness markers. The typical pictures of sphere formation assays from 97H-overexpression and Huh7-overexpression cells (C). Flow cytometric analysis of CD133+ cells in 97H-overexpressing and Huh7-overexpressing cells (D). *P < 0.05, **P < 0.01.
MTDH downregulation inhibited HCC stem cell phenotypes. MTDH, CD133, Nanog, and Oct4 mRNA expression levels in 97H and Huh7-RNAi (A). In comparison with MTDH-LV-RNAi, CD133 and Nanog level of protein expression were elevated in MHCC-97H-NC and Huh7-NC cells (B). The typical pictures of sphere formation assays from 97H-RNAi and Huh7-RNAi cells (C). Figures 97H-LV and Huh7-LV immunofluorescence images of Nanog (red) (D). DAPI (blue) was used to stain the nuclei. *P< 0.05, **P < 0.01, ***P < 0.001.
**Figure 6**

**In vivo, MTDH stimulated tumorigenesis in HCC.** Tumors derived from nude mice injected with 97H-RNAi (n=3), 97H-NC cell(n=3) (A). Tumor volume (B) showed that inhibition of MTDH significantly inhibited tumor growth. Representative IHC images of tumours from nude mice stained with CD133, MTDH. (Scale bars = 200 μm). Histograms show the IHC score. **P < 0.01, ***P < 0.001.
**Figure 7**

**Infiltration of immune cells in TCGA samples using ssGSEA.** Immune cell infiltration between LIHC samples and normal samples (A). Different immune cell infiltration patterns in high and low expression samples of MTDH (B).
Immune infiltration and MTDH expression levels. (A). MTDH expression levels correlated with immature dendritic cells ($r = 0.28$, $p < 0.0001$) (Fig8 B), Th2 cells ($r = 0.26$, $p < 0.0001$) (Fig8 C), memory B cells ($r = 0.25$, $p < 0.0001$) (Fig8 D), and central memory CD4 T cells ($r = 0.22$, $p < 0.0001$) (Fig8 E) expression was positively correlated. The levels of MTDH expression correlated negatively with activated CD8 T cell ($r$...
= -0.23, \ p < 0.0001) (Fig 8 F), eosinophils \ (r = -0.13, \ p < 0.05) (Fig 8 G), activated B cells \ (r = -0.12, \ p < 0.05) (Fig 8 H), monocytes \ (r = -0.12, \ p < 0.05) (Fig 8 I) expression was negatively correlated.

**Figure 9**

**Correlation between MTDH expression levels and immunomodulators.** Heat map shown the correlation between MTDH and immunosuppressive agents (A) and immunostimulants (B) in hepatocellular carcinoma. Correlations between MTDH and MICB \ (r = 0.207, \ p = 5.76e-05)(Fig 9 C, NT5E \ (r = 0.201, \ p = 9.8e-05) Fig 9 D, and TNFSF14 \ (r = 0.143, \ p = 0.00576)(Fig 9 E).
Figure 10

Chemokine and chemokines receptor expression correlated with MTDH expression. An analysis of MTDH and chemokines (A) and receptors (B) in LIHC is presented as a heat map. CXCL2 (r =0.224, p=1.34e-05) and CX3CL1 (r =0.245, p=1.73e-06) were positively correlated with MTDH expression levels (Fig10 C) and negatively correlated with CXCL12 (r =0.208, p=5.4e-05) (Fig10 E).

Supplementary Files

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- Supplementarymaterial.docx
- Flowdiagramofthestudy.tif